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Antibodies to the Leucine-Rich Repeat Region of Internalin Block Entry of *Listeria monocytogenes* into Cells Expressing E-Cadherin

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Internalin, a surface protein essential for entry of *Listeria monocytogenes* EGD into epithelial cells, was used as an antigen to raise nine monoclonal antibodies. These monoclonal antibodies recognized seven distinct epitopes which were located in three different regions of the protein. Three of them inhibited internalin-mediated entry and recognized the amino-terminal leucine-rich repeat region of the protein, suggesting that this region is essential for entry.

Listeria monocytogenes is a gram-positive bacterium responsible for severe food-borne infections affecting primarily pregnant women or immunocompromized hosts. This facultative intracellular pathogen is able to enter, survive, and multiply in a wide variety of phagocytic and nonphagocytic cells, both in vivo and in vitro (15). Entry into epithelial cells requires expression of internalin, a bacterial surface protein which interacts with E-cadherin on the mammalian cell (13).

Internalin is an 800-amino-acid protein with a signal sequence and a carboxy-terminal cell wall sorting signal that are required for its export to the bacterial surface and its association with the cell wall peptidoglycan, respectively (5, 8, 11). In addition, internalin has two regions of tandemly repeated sequences. The first region is made of 15 22-amino-acid leucine-rich repeats (LRRs). The second region is formed of three consecutive repeats (B repeats), two of 70 amino acids and one of 49 amino acids.

In order to identify regions of internalin essential for the entry process, we raised monoclonal antibodies (MAbs) against internalin, mapped the protein regions recognized by these MAbs, and tested the ability of the different MAbs to inhibit internalin-mediated entry into cells.

Production and characterization of nine anti-internalin MAbs. Internalin was purified from bacterial culture supernatant of a recombinant *Listeria innocua* strain as previously described (13) and used as an antigen for the production of MAbs. Nine MAbs were obtained. Their properties are summarized in Table 1. K_d values were determined for each of the MAbs by an enzyme-linked immunosorbent assay (7). The nine MAbs have affinities ranging from 1.4×10^{-8} M for B11.6 to 4.6×10^{-11} M for G6.1. Affinities are equivalent for native internalin and internalin denatured for 5 min at 100°C in 0.5 M Tris-HCl–5% sodium dodecyl sulfate (SDS)–2% β-mercaptoethanol, suggesting that the epitopes recognized by the nine antibodies are not strongly dependent on the protein conformation. A previously described (14) antibody competition assay was used to measure the ability of the different MAbs to compete for the same binding site on the antigen. Based on this assay, the nine MAbs can be classified in seven specificity groups, J14.1 and D18.6 having the same specificity as K18.4 and C20.4, respectively. In Western blot (immunoblot) experiments, the nine MAbs, when used at a concentration of 1 μg/ml, detected purified internalin as well as internalin present in recombinant *Escherichia coli* strains or *L. monocytogenes* EGD crude extracts. The strongest signal was obtained with K18.4, and the weakest was obtained with B11.6. Specificity of the nine MAbs for internalin was tested in Western blot experiments by using crude extracts of strain EGDΔ*inlA* as a control. This strain has an in-phase internal deletion in the *inlA* gene and thus produces an 18-kDa internalin variant InlAΔ (LRRs-B repeats). Under the conditions used, apart from InlAΔ(LRRs-B repeats) detected only with C20.4 and D18.6 (see below), no other cross-hybridizing protein was detected with any of the nine MAbs in EGDΔ*inlA* crude extracts (data not shown), indicating that the nine MAbs are specific for internalin and/or that the homologous proteins encoded by the internalin gene family are not or only poorly expressed.

Epitope mapping: the MAbs recognize three different regions of internalin. Several internalin variants, either purified or present in bacterial extracts, were used to map the epitopes recognized by the MAbs (Fig. 1A). In addition to internalin purified from *L. innocua* (*inlA*) culture supernatants, we used an internalin variant lacking its cell wall anchor (InlAΔcwa) and purified from culture supernatants of strain EGDΔ*inlA* (*inlA*Δ*cwa*) (11). We also used the in-frame deleted strain EGDΔ*inlA*, which only expresses the 18-kDa internalin variant InlAΔ(LRRs-B repeats). Finally, we constructed three new bacterial strains expressing internalin variants. Plasmids and strains expressing these variants are presented in Table 2.

The first internalin variant, InlAΔLRRs, corresponds to an internalin deleted for its LRRs (amino acids 74 to 413). It was constructed from plasmid pPE10 (11) by deleting the 1,020-bp *Bgl*II-*Bcl*I fragment internal to the *inlA* gene and encoding the LRR region (positions 1751 to 2771 [8]). Plasmid pPE10 was digested with *Bgl*II and *Bcl*I, two restriction enzymes generating compatible ends. Since a second *Bcl*I site is present in the *inlA-inlB* intergenic region (our unpublished results), we first linearized pPE10 with *Bgl*II, performed a partial *Bcl*I digestion, and gel purified the plasmids cut at the *Bcl*I site located

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TABLE 1. Properties of anti-internalin murine MAbs

MAb[a]	Native K_d (M)	Denatured K_d (M)	Epitope grouping[b]	Inhibitory activity[c]	Detection of purified internalin by Western blot after non-denaturing PAGE	Detection of internalin by indirect immunofluorescence labeling
G6.1	4.6×10^{-11}	2.3×10^{-11}	A	+	±	++
I4.4	1.9×10^{-10}	2.5×10^{-10}	B	+	±	++
L7.7	7.3×10^{-11}	2.7×10^{-11}	C	+	+	++
B11.6	1.4×10^{-8}	1.2×10^{-7}	D	−	−	−
F20.23	3.0×10^{-10}	3.8×10^{-10}	E	−	−	−
J14.1	1.7×10^{-10}	8.8×10^{-11}	F	−	±	+
K18.4	4.0×10^{-11}	3.2×10^{-11}	F	−	+	+
D18.6	3.2×10^{-10}	1.6×10^{-10}	G	−	−	−
C20.4	7.0×10^{-10}	1.4×10^{-10}	G	−	−	−

[a] The isotype for all MAbs was immunoglobulin G1.
[b] Letters correspond to groups of specificity, as determined by competition assays.
[c] Inhibitory effect on entry of *L. innocua* (*inlA*) into S180(L-CAM2) cells.

in the *inlA* gene, which were finally religated and used to transform *E. coli* MC1061, giving rise to plasmid pPE13.

The second internalin variant, InlAΔB repeats, corresponds to an internalin deleted for its B-repeat region (amino acids 518 to 706). It was constructed from plasmid pPE10 by replacing the 601-bp *Bst*XI-*Pfl*MI fragment internal to the *inlA* gene and encoding the B-repeat region (positions 3061 to 3663 [8]) by an adaptor obtained by annealing oligonucleotide 5′-GTG GAAGCTGGGAATCCTGTGGCACCACCAACAA-3′ with oligonucleotide 5′-TTGGTGGTGCCACAGGTTACCCAGC TTCCACTTCT-3′. Annealing was performed by mixing equimolar amounts of the oligonucleotides, incubating for 10 min at 65°C, and cooling slowly to room temperature. Annealed oligonucleotides were ligated to plasmid pPE10 digested with *Bst*XI and *Pfl*MI to generate plasmid pPE14.

The third internalin variant, the LRR fragment, corresponds to the amino-terminal part of internalin including the LRRs (amino acids 1 to 423). It was constructed by amplifying by PCR a 2-kb DNA fragment from plasmid pPE10 with primers OML1 (5′-AGACGTCAACGTAAACG-3′) and OML2 (5′-A TAGGTACCTCAGTTACTGGTGCATTTGT-3′) derived from the *inlAB* locus (positions 801 to 817 and 2800 to 2783, respectively [8]) with an additional *Kpn*I restriction site in OML2. The amplified fragment was gel purified, digested with *Hin*cII and *Kpn*I, and ligated with plasmid pUC18 digested by *Hin*cII and *Kpn*I, yielding plasmid pPE15.

In *E. coli* strains transformed with plasmid pPE13, pPE14, or pPE15, each of the three internalin variants was expressed with the expected molecular mass, as shown in Western blot experiments with internalin-specific MAbs (Fig. 1B).

The nine MAbs were tested in Western blot experiments by using the two purified proteins (internalin or InlAΔcwa), crude bacterial extracts from *Listeria* strain EGDΔ*inlA*, or crude bacterial extracts from *E. coli* strains expressing the three internalin variants. Results are given in Fig. 1C. The epitopes recognized by the nine MAbs cover the whole protein. G6.1, I4.4, and L7.7 recognized the amino-terminal part of the protein. B11.6, F20.23, J14.1, and K18.4 recognized the central region of internalin and, finally, C20.4 and D18.6 recognized an epitope localized in the carboxy-terminal pre-anchor region of internalin (positions 713 to 759). These results are in agreement with the epitope grouping based on the antibody competition assay (see Table 1).

Three MAbs blocked bacterial entry into cells. In order to identify the regions of internalin playing a crucial role in entry into mammalian cells, we tested the ability of the different MAbs to inhibit entry of a recombinant *L. innocua* strain expressing internalin into a fibroblastic cell line, S180(L-CAM2), expressing the chicken homolog of E-cadherin (L-CAM). In these conditions, entry is strictly internalin- and E-cadherin-dependent (13). Invasion assays were performed in 24-well plates as previously described (6) except that 5 μg of MAb per ml was added to bacteria (10^7 CFU/ml in Dulbecco's modified Eagle's medium) 30 min before and during the 1-h cell infection step. Three MAbs, G6.1, I4.4, and L7.7, had an inhibitory effect on entry (Fig. 2). There were 55-fold, 27-fold, and 2.5-fold reductions in entry with G6.1, I4.4, and L7.7, respectively, compared with entry of bacteria in the absence of a MAb. Inhibition was not significantly increased when a 50-μg/ml concentration of MAb was used; it was not observed when a 0.5-μg/ml concentration of MAb was used (data not shown). Inhibition was specific since no inhibition was observed with the other MAbs or with rabbit immunoglobulin G when used at identical final concentrations. MAbs G6.1, I4.4, and L7.7 were also the only MAbs able to inhibit entry of *L. monocytogenes* EGD into Caco-2 cells, with observed reductions of entry of 11-fold, 8-fold, and 3-fold, respectively (data not shown). In that case, inhibition was not as dramatic as that observed with the *L. innocua* (pP1A) strain in S180(L-CAM2) cells, in agreement with the previous observation that entry of EGD into Caco-2 cells is not strictly internalin dependent (13).

These data thus suggest that the amino-terminal LRR region of internalin is essential for entry, in agreement with the surface exposure of the LLR region. Indeed, antibodies to this region give a strong signal when used in immunofluorescence or immunogold labeling of intact bacteria (Table 1) (11). This interpretation is also in agreement with the previous observation that invasiveness correlates with the amount of epitopes accessible to G6.1 MAb on the bacterial cell surface (11). However, not all surface-exposed regions of internalin seem to play a role in entry, since MAbs L7.7 and K18.4, which give the strongest signals in immunogold labeling of intact bacteria (data not shown) and thus are directed against two different exposed regions of internalin, have, respectively, little or no inhibitory activity on entry. Our results indicate that the LRR region of internalin is a good candidate to interact directly with the E-cadherin cellular receptor molecule. A role in entry of other parts of the internalin molecule cannot be excluded since antibodies to the LRR region could have steric effects on other parts of the molecule.

Residues of the LRR region that would interact with E-cadherin are not known and could not be deduced from sequence comparison of internalin with the two other known E-cadherin ligands (3, 10). E-cadherin itself is a ligand for

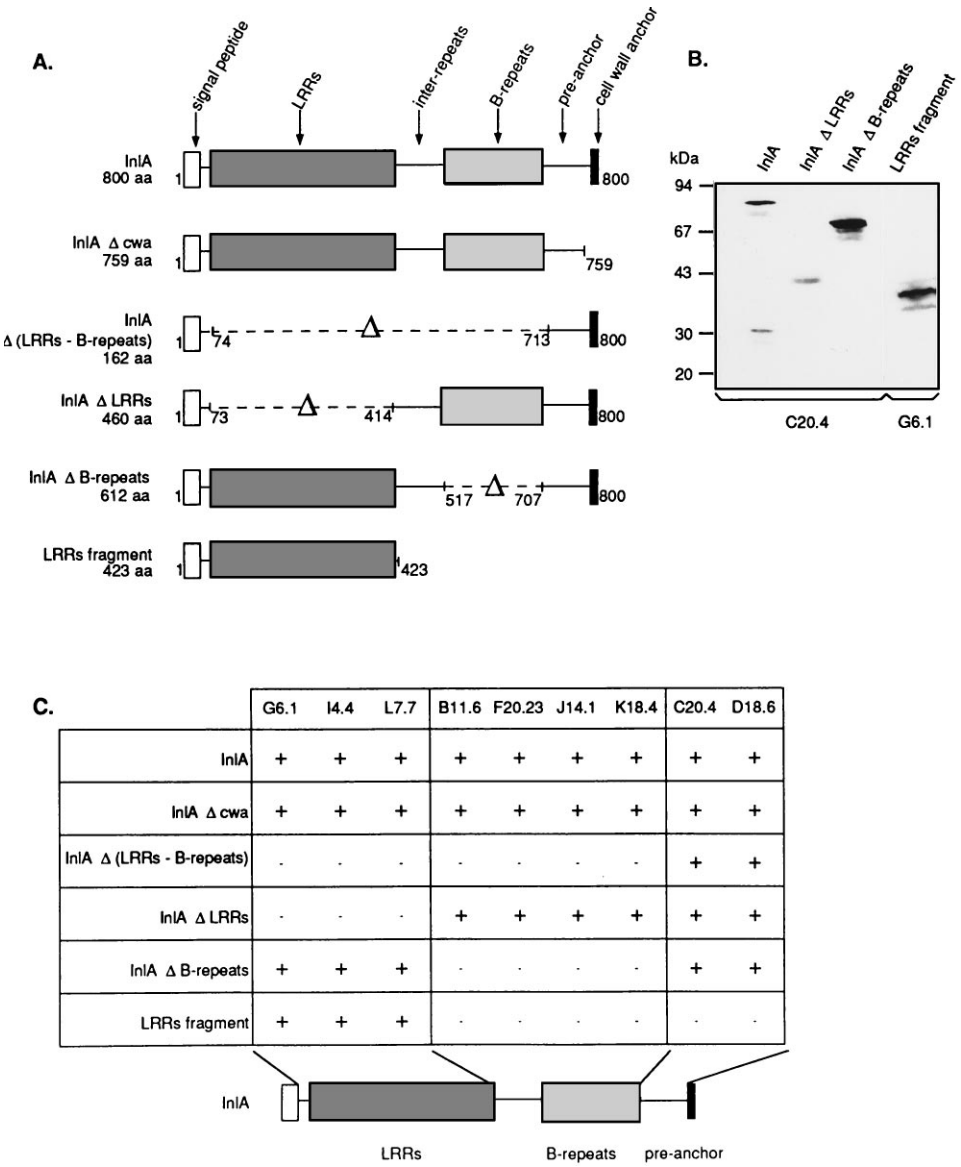

FIG. 1. Mapping of internalin regions recognized by the MAbs. (A) Schematic representation of internalin and its variants. Wild-type internalin (InlA) is divided into six regions: the signal peptide (positions 1 to 35), the LRR region (positions 73 to 423), the interrepeat region (positions 424 to 517), the B-repeat region (positions 518 to 706), the pre-anchor region (positions 707 to 766), and the cell wall anchor (positions 767 to 800). The various internalin variants are presented. The length of each internalin variant, including the signal peptide and the carboxy-terminal cell wall anchoring signal if present, is given. aa, amino acids. (B) Example of a Western blot analysis of internalin variants expressed in *E. coli*. Crude extracts of *E. coli* expressing InA, InlAΔLRRs, InlAΔB repeats, or the LRR fragment were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. Internalin variants were detected with MAb C20.4 except for the LRR fragment, which was detected with MAb G6.1. Positions of the molecular mass markers are indicated on the left. (C) Epitope mapping (summary). Western blot experiments were performed with purified internalin and crude extracts of strain MC1061(pPE10) (InlA); InlAΔcwa purified from strain EGDΔ*inlA* (InlAΔcwa); crude extracts of EGDΔ*inlA* [InlAΔ(LRRs-B repeats)]; and crude extracts of strains MC1061(pPE13), MC1061(pPE14), and MC1061(pPE15) (InlAΔLRRs, InlAΔB repeats, and LRRs, respectively). Regions of internalin and internalin variants recognized by each of the nine MAbs are indicated.

E-cadherin and it is through homophilic E-cadherin–E-cadherin interactions that this protein mediates cell-cell contact. These interactions involve the amino-terminal extracellular domain that contains a "cell adhesion recognition" sequence. This type of sequence is present in several cell and substrate adhesion molecules and is generally composed of at least three amino acid residues. In the case of E-cadherin, it includes the highly conserved tripeptide HAV (1). No HAV sequence is found in internalin, and E-cadherin and internalin do not share any similarities. $\alpha^E\beta_7$ integrin present on the surface of intraepithelial lymphocytes is the second heterophilic ligand for E-cadherin (3, 10). In this case also, no sequence homologies between internalin and $\alpha^E\beta_7$ integrin can be detected. Absence of sequence homologies between internalin, E-cadherin, and $\alpha^E\beta_7$ integrin thus suggests that either internalin binds E-cadherin at a site different from the two other ligand binding sites or that internalin has structural similarities with the other ligands that cannot easily be identified from primary sequence comparisons. Interestingly, a similar situation was observed with the *Yersinia pseudotuberculosis* invasin, another bacterial

TABLE 2. Plasmids and bacterial strains

	Genotype or relevant properties	Source or reference
Plasmids		
pUC18	AmpR	16
pPE10	pUC18 derivative carrying *inlA*	11
pPE13	pPE10 derivative encoding InlAΔLRRs	This work
pPE14	pPE10 derivative encoding InlAΔB repeats	This work
pPE15	pPE10 derivative encoding LRRs	This work
E. coli strains		
MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*) *7679* Δ*lacX74 galU galK rpsL thi*	2
BUG1293	MC1061 (pPE10)	11
BUG1381	JM101 (pPE10)	This work
BUG1344	MC1061 (pPE13)	This work
BUG1345	MC1061 (pPE14)	This work
BUG1380	MC1061 (pPE15)	This work
Listeria strains		
EGD	Wild type, *L. monocytogenes*	12
BUG947	EGD Δ*inlA*	4
BUG1290	EGD Δ*inlA* (*inlA*Δ*cwa*)	11
BUG991	*L. innocua* (pP1A)	4

surface protein mediating entry into cells. Indeed, this protein mediates bacterial entry by interacting with β1-integrin, its cellular receptor. However, the tripeptide RGD present in fibronectin, a classical integrin ligand and involved in the fibronectin-integrin interaction, is not present in invasin although invasin and fibronectin bind to the same site on integrin (9).

Structural analysis of internalin and its LRR region as well as the identification of residues recognized by the three inhibiting MAbs should clarify the internalin–E-cadherin interactions and their consequences for entry and the whole infectious process.

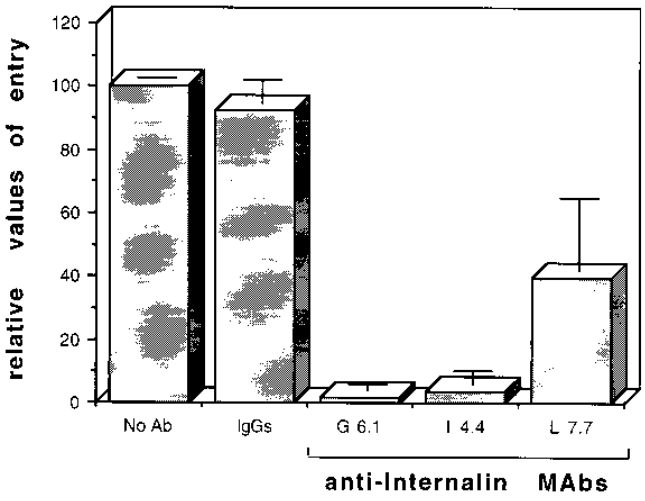

FIG. 2. Inhibitory effect of three MAbs on bacterial entry into cells. This graph summarizes three independent invasion assays of *L. innocua* expressing internalin in S180(L-CAM2) fibroblastic cells expressing L-CAM, the chicken homolog of E-cadherin (13). MAbs G6.1, I4.4, and L7.7 or rabbit immunoglobulins G (IgGs) were added to the bacterial inoculum at a concentration of 5 μg/ml, 30 min before and during the infection step. Values along the vertical axis are given relative to the percentage of invasion measured under the same conditions in the absence of antibodies and arbitrarily fixed to 100. Invasion assays were performed in the absence of serum. No bactericidal activity of MAb G6.1, I4.4, or L7.7 was detected (data not shown).

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